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In situ recovery of lycopene during biosynthesis with recombinant *Escherichia coli*

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ABSTRACT

Lycopene is produced by recombinant *Escherichia coli* expressing genes to encode for the lycopene biosynthesis. However, the productivity of lycopene seemed to be limited by many factors including product toxicity. In the present study, we have investigated physiology of recombinant *E. coli* during biosynthesis and *in situ* recovery of lycopene based on an organic/aqueous two-phase system. Lycopene, the 40-carbon molecule product, was little extracted from recombinant *E. coli* cells to octane or decane phase. However, partial digestion of cell walls with lysozyme promoted extraction of lycopene into the organic phases. Engineering of an organic/aqueous two-phase system allowed recombinant *E. coli* cells to produce ca. 40% larger amount of lycopene compared to that in a conventional aqueous single-phase system. Optimization of the *in situ* product recovery process will lead to further increase of product concentration and productivity.

1. Introduction

Overproduction of isoprenoid-based molecules such as carotenoids and terpenoids is of significant interest due to a variety of biological activities as food colorants, flavor compounds, and pharmaceutical intermediates (Chang and Keasling, 2006; Sandmann, 2001). However, it is very difficult to produce carotenoids and terpenoids to a high concentration because of the structural complexity to require multisynthetic pathways and the high toxicity towards whole-cell biocatalysts. Carotenoids and terpenoids may accumulate in the cellular membrane bilayer and destruct the membrane integrity (Sikkema et al., 1995; Sung et al., 2007), resulting in a significant damage to the function of the membranes as a matrix for embedded proteins and as a selective barrier.

Rapid development of systems biology and synthetic biology allows us to overcome many problems, which resulted from complexity of the biosynthetic pathways (Das et al., 2007; Klein-Marcuschamer et al., 2007; Withers and Keasling, 2007). As a result, a number of carotenoids and terpenoids (e.g., lycopene) have been shown to be efficiently produced by engineered microorganisms (Alper et al., 2006; Jin and Stephanopoulos, 2007; Yoon et al., 2006).

Attenuation of toxicity of hydrophobic organic products towards biocatalysts has been achieved by *in situ* product recovery, which was often based on extraction into organic solvents (Fujiwara et al., 2008; Park, 2007; Stark and von Stockar, 2003). For instance, (*S*)-styrene oxide (Park et al., 2006) and 3,4-dimethylbenzaldehyde (Bühler et al., 2003) were shown to be produced at a rate of ca. 60 U/g CDW (3.6 mmol/(g(CDW) h)). However, productivities of large molecules such as 4-androstene-3,17-dione ($C_{19}H_{26}O_2$) at an organic/aqueous two-liquid phase system remained below 1 U/g CDW (Staebler et al., 2004).

In the present study, we examined the physiology of recombinant *Escherichia coli* BL21(DE3) (pT-GLYC4, pSdxs) during biosynthesis of lycopene as well as *in situ* product recovery by using an organic/aqueous two-phase system with an aim to increase amount of lycopene produced per g cell mass.

2. Materials and methods

2.1. Cultivation and biotransformation

The recombinant *E. coli* DH5α (pT-GLYC4, pSdxs) and BL21(DE3) (pT-GLYC4, pSdxs) strains were cultivated in 2YT medium (16 g l^{-1} tryptone, 10 g l^{-1} yeast extract, 5.0 g l^{-1} NaCl) containing 2.0 g l^{-1} arabinose and 5.0 g l^{-1} glycerol at 29 °C on a rotary shaker (Jeio Tech, Korea) at 250 rpm. The pT-GLYC4 was constructed by introducing *gps* of *Archaeoglobus fulgidus*, *crtB* and *crtI* of *Pantoea agglomerans*, and *idi* of *E. coli* into pTrc99A (Yoon et al., 2007a). The pSdxs was

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originated from pSTV28 containing *dxs* of *E. coli* (Yoon et al., 2007b). Chlorampnenicol (50 μg ml^{-1}) and ampicillin (100 μg ml^{-1}) (both from Sigma–Aldrich, USA) were added as required. Biotransformation was initiated with leaky expression of the promoters (i.e., *trc* and *lac* promoters of pT-GLYC4 and pSdxs, respectively) in the 2YT medium. When the cultivation and biotransformation were carried out in a bioreactor (KoBiotech, Korea), the pH was kept at pH 7.0 by feeding ammonia solution. Agitation speed and aeration rate were set at 600 rpm and 2.5 vvm, respectively.

2.2. Preparation of spheroplasts

Spheroplasts of the recombinant *E. coli* BL21(DE3) were prepared as has been reported in an earlier study (Birdsell and Cota-Robles, 1967; Sullivana et al., 2005); *E. coli* cells were collected by centrifugation, washed with 10 mM potassium phosphate buffer (pH 7.0), and resuspended into a 0.5 M sucrose solution made in 10 mM potassium phosphate buffer. Lysozyme (Sigma–Aldrich, USA) was added to the cell suspension at a final concentration of 50 μg ml^{-1}. After incubation in a 37 °C water bath for 2 h, the suspension was diluted 1:1 with 10 mM potassium phosphate buffer. EDTA was added to a final concentration of 10 mM. After 10 min incubation, the resulting spheroplasts were collected by centrifugation at 500 × *g* for 15 min.

2.3. Analysis of metabolites and lycopene

Concentrations of arabinose, glycerol, and acetic acid were measured by high performance liquid chromatography (HPLC) (Waters, USA) equipped with an HPX-87H column (Bio-Rad Aminex, USA). The 5 mM H_2SO_4 solution flowed into the column at a rate of 0.6 ml min^{-1} at 45 °C. Quantification of lycopene was carried out according to the method reported in our previous study (Yoon et al., 2006).

3. Results and discussion

3.1. Effect of host strains on the biosynthesis of lycopene

Lycopene was produced by *E. coli* DH5α (pT-GLYC4, pSdxs) and BL21(DE3) (pT-GLYC4, pSdxs) strains in the 2YT medium containing 2.0 g l^{-1} arabinose and 5.0 g l^{-1} glycerol at 29 °C (Fig. 1). Lycopene accumulated in the biomass, most probably in the lipid bilayer while cell growth was maintained. The content of lycopene in dry cell mass was similar in both strains ranging 2.6–2.9 mg g^{-1}, which resulted in a final lycopene concentration of about 11.8 mg l^{-1}. However, the production rate of lycopene was approximately twofold greater in the recombinant *E. coli* BL21(DE3) as compared to that of the recombinant *E. coli* DH5α. The reasons for the fast production of lycopene by the recombinant *E. coli* BL21(DE3) strain remained to be investigated.

3.2. Cell physiology during biosynthesis of lycopene

With an aim to understand the factors that influence the lycopene biosynthesis of recombinant *E. coli*, biotransformation was carried out in a bioreactor, which allows to control pH and to monitor DOT of the reaction medium (Fig. 2). Agitation speed and aeration rate were set at 600 rpm and 2.5 vvm, respectively.

The overall trend of biotransformation by *E. coli* BL21(DE3) (pT-GLYC4, pSdxs) was very similar to that of biotransformation shown in Fig. 1B. Lycopene accumulated in the dry cell mass to a concentration of around 2.3 mg g^{-1}, resulting in a final concentration of 10.2 mg l^{-1}. Arabinose was first consumed as a carbon source generating acetic acid. Afterwards, glycerol and acetic acid were simultaneously consumed, maintaining further cell growth and lycopene biosynthesis. Interestingly, the DOT dropped to around 60% at t = 5 h and gradually rose even with no depletion of carbon source in the culture medium. This indicates that oxygen uptake rate of cells or metabolic activity began to decrease from that time. Cessation of cell growth was followed by termination of the biosynthesis of lycopene (t = 9 h). These results indicate that carbon metabolism and growth of the *E. coli* BL21(DE3) (pT-GLYC4, pSdxs)

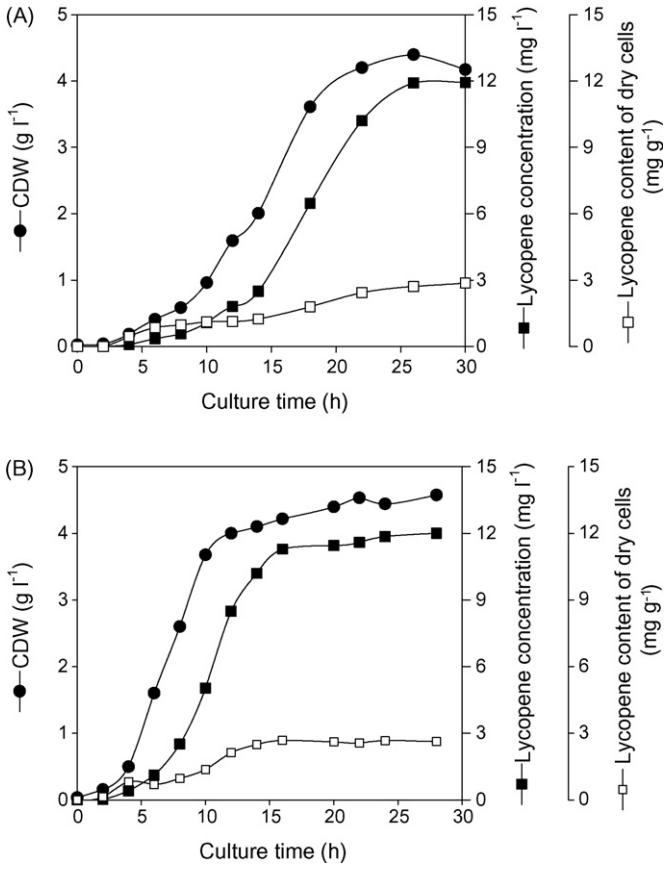

Fig. 1. Effect of host strains on the biosynthesis of lycopene. Lycopene was produced by *Escherichia coli* DH5α (pT-GLYC4, pSdxs) (A), and *E. coli* BL21(DE3) (pT-GLYC4, pSdxs) (B) in 2YT medium containing 2.0 g l^{-1} arabinose and 5.0 g l^{-1} glycerol at 29 °C on a rotary shaker at 250 rpm.

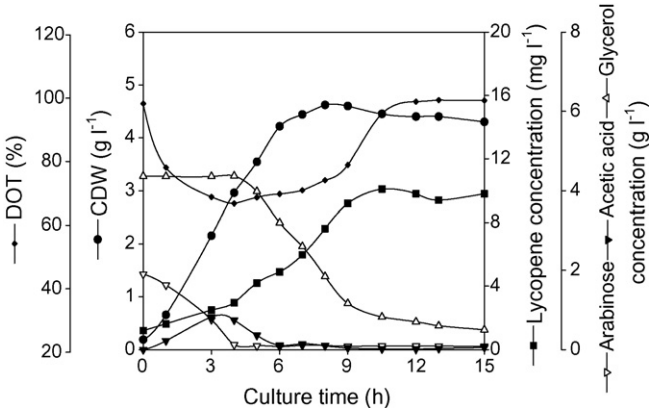

Fig. 2. Biosynthesis of lycopene in a 5 l reactor. Lycopene was produced by *E. coli* BL21(DE3) (pT-GLYC4, pSdxs) in 2YT medium containing 2.0 g l^{-1} arabinose and 5.0 g l^{-1} glycerol at 29 °C. Agitation speed and aeration rate were set at 600 rpm and 2.5 vvm, respectively.

were repressed or inhibited by some factors, which had been generated during biosynthesis of lycopene. This appears in turn to repress the biosynthesis of lycopene.

One of the factors, which influence metabolic activity of the recombinant cells during biosynthesis of lycopene may include product toxicity. Lycopene has been reported to cause significant damage to the cytoplasmic membrane and to inhibit the growth of *Candida albicans* at a concentration over 5 mg l^{-1} (Sung et al., 2007). Therefore, it would be necessary to attenuate product toxicity to reach high concentration of lycopene with the recombinant strains.

3.3. *In situ* recovery of lycopene during biosynthesis

In order to attenuate the presumed toxicity of lycopene towards the microbial cells, we tried to extract lycopene from biomass during biosynthesis by using an organic/aqueous two-phase system.

When octane or decane was added into the culture of *E. coli* BL21(DE3) (pT-GLYC4, pSdxs) at the early stationary phase to a phase ratio of 0.5, only small amount of lycopene was observed in the organic solvents (data not shown). This may be due to bulkiness of lycopene molecule, which molecular weight is 536, around fivefold of those of the compounds, which were reported to be extracted from biomass into organic solvents (Park, 2007; Stark and von Stockar, 2003).

In order to facilitate extraction of lycopene from the cell mass, interaction between organic solvents and lycopene in the biomass was elevated; the recombinant cells were subject to the reaction with lysozyme and EDTA to remove outer cellular membrane and peptidoglycan layer of cell wall. Without treatment of lysozyme and EDTA, lycopene molecules that existed in the biomass were little extracted into the organic phase (Fig. 3A and C). However, treatment of lysozyme and EDTA allowed larger amount of lycopene extracted from the biomass to the organic phase (Fig. 3B and D). This result indicates that digestion of cell wall promoted extraction of lycopene from the biomass.

Another interesting point was that $OD_{600\,nm}$ of the aqueous medium increased during biotransformation at the decane/aqueous system, whereas $OD_{600\,nm}$ decreased markedly at the octane/aqueous system (Fig. 3B and D). The reduction of OD pointing to lysis of cells was probably due to toxicity of octane to the lysozyme and EDTA-treated cells; log *P* (the logarithm of the ratio of the concentrations of a compound between water and octanol) of octane is 4.5, which is 25% lower than decane. Therefore, decane is more suitable as the extraction solvent of lycopene.

The 0.25 M sucrose solution, which was used as the medium during preparation of the spheroplasts (Birdsell and Cota-Robles, 1967), was compared with 2YT medium as the aqueous phase. When the decane/sucrose solution was used as the reaction medium, $OD_{600\,nm}$ of the aqueous phase remained rather constant and 43% larger amount of lycopene was extracted into the organic phase (data not shown). This result indicates that the decane/sucrose system allowed the *E. coli* cells to keep

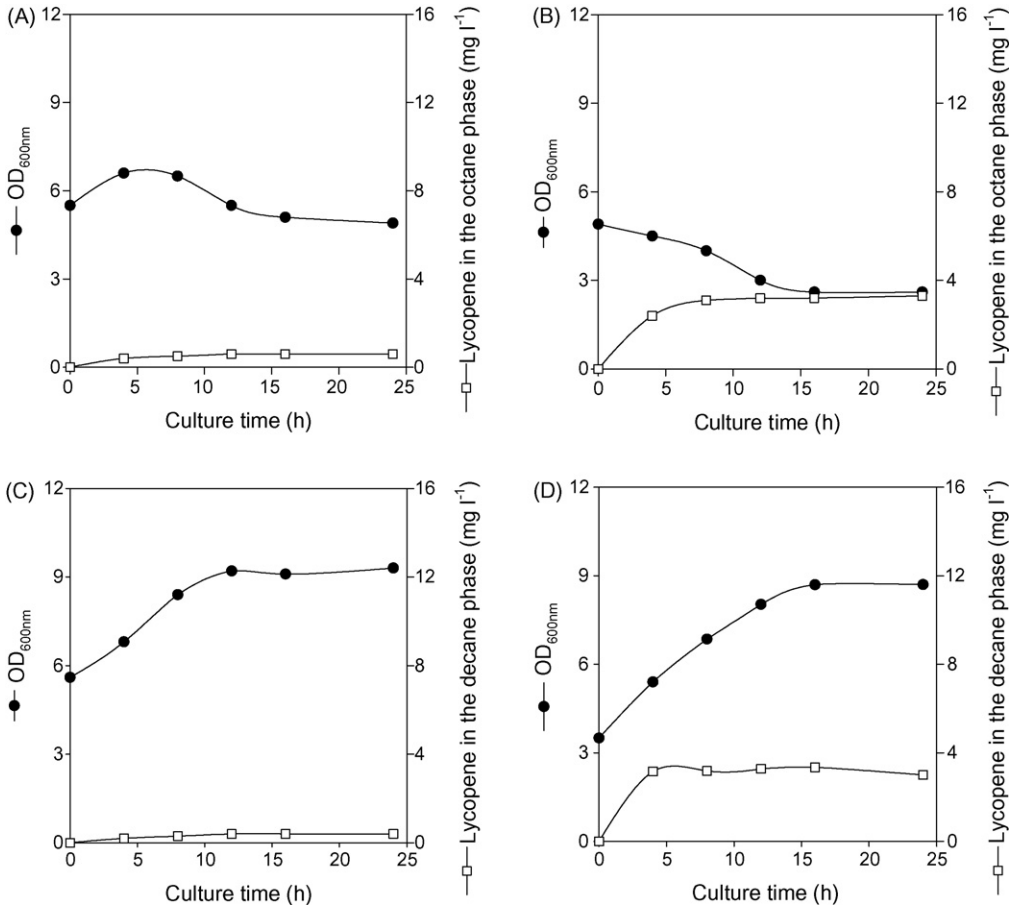

Fig. 3. *In situ* recovery of lycopene during biosynthesis. The lycopene biosynthesis was carried out in the presence of octane (A and B) or decane (C and D) at a phase ratio of 0.5. (B) and (D) show the experiments done with spheroplasts, which were prepared as follows; *E. coli* BL21(DE3) (pT-GLYC4, pSdxs) cells were harvested by centrifugation at the early stationary phase of batch culture, treated with lysozyme and EDTA at the phosphate buffer (see Section 2 for details), and resuspended into the 2YT medium. (A) and (C) show the biotransformations by cells without treatment of lysozyme and EDTA.

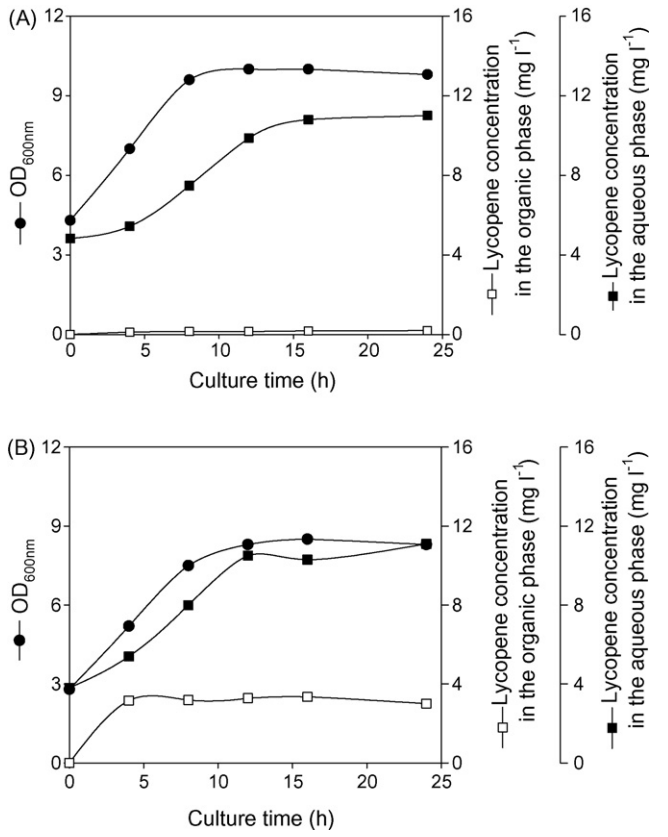

Fig. 4. Biosynthesis of lycopene in the decane/aqueous system (phase ratio of 0.5). The biotransformation was carried out by cells which were not treated (A) or were treated (B) with lysozyme and EDTA. The reaction conditions with lysozyme and EDTA were the same as in Fig. 3.

spheroplasted and thereby allowed more lycopene extracted into the organic phase. Increase of $OD_{600\,nm}$ at the decane/2YT medium (Fig. 3D) indicates that the spheroplasted cells returned to the intact state during biotransformation.

In a separate experiment, effect of *in situ* recovery of lycopene on the biosynthesis of recombinant *E. coli* was investigated (Fig. 4). The *E. coli* BL21(DE3) (pT-GLYC4, pSdxs) cells, which were not treated with lysozyme and EDTA, produced 11.2 mg(lycopene) l^{-1} of the 2YT medium, which was sum of amount of lycopene present in the aqueous phase and of lycopene extracted into the organic phase. On the other hand, the total amount of lycopene produced by the recombinant *E. coli* cells, which were treated with lysozyme and EDTA, reached 14.1 mg l^{-1} of the aqueous medium. The overall amount of lycopene produced per g dry cells was 3.8 mg, which is 42% larger compared to that of control cells (lysozyme and EDTA non-treated cells).

This study shows that lycopene, the 40-carbon molecule product, could be efficiently extracted from biomass into organic solvents after treatment with lysozyme and EDTA. Engineering of an organic/aqueous two-phase system allowed recombinant *E. coli* cells to produce ca. 40% larger amount of lycopene. Optimization of the *in situ* product recovery process will lead to further increase of product concentration and productivity.

Acknowledgment

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